Metagenomics reveals diet-specific specialization in fungus gardens of grass- and dicot-cutter ants

Lily Khadempour[1,2,3], Huan Fan[1], Ken Keefover-Ring[4], Camila Carlos[1,3], Nilson S. Nagamoto[5], Miranda A. Dam[6], Monica T. Pupo[7], Cameron R. Currie[1,3]

Affiliations:

[1] Department of Bacteriology, University of Wisconsin-Madison, Madison, WI, USA, 53706

[2] Department of Integrative Biology, University of Wisconsin-Madison, Madison, WI, USA, 53706

[3] Department of Energy Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI, USA, 53706

[4] Departments of Botany and Geography, University of Wisconsin-Madison, Madison, WI, USA, 53706

[5] Department of Plant Protection, UNESP – São Paulo State University, Botucatu, SP, Brazil, CEP: 18603-970

[6] Department of Nutritional Sciences, University of Wisconsin-Madison, Madison, WI, USA 53706

[8] School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, CEP: 14040-903

Conceived of the project (L.K., C.R.C), performed computational and statistical analysis (L.K., H.F., C.C.), analyzed data (L.K.) designed sampling methodology (L.K.) performed lab work (L.K., M.A.D., K.K.), performed field work (L.K., N.S.N.) provided resources and facilities (M.T.P., C.R.C) and wrote the manuscript (L.K., H.F., K.K., C.R.C.).

Abstract

Leaf-cutter ants in the genus *Atta* are dominant herbivores in the Neotropics. While most species of *Atta* cut dicots to incorporate into their fungus gardens, some species specialize on grasses. Here we examine the bacterial community associated with the fungus gardens of grass- and dicot-cutter ants to elucidate the potential role of bacteria in leaf-cutter ant substrate specialization. We sequenced the metagenomes of 12 *Atta* fungus gardens, across four species of ants, with a total of 5.316 Gbp of sequence data. We show significant differences in the fungus garden bacterial community composition between dicot- and grass-cutter ants, with grass-cutter ants having significantly lower diversity and a significantly higher abundance of *Pantoea*. Reflecting this difference in community composition, the bacterial functional profiles between the fungus gardens are significantly different. Specifically, grass-cutter ant fungus garden metagenomes are particularly enriched for genes responsible for amino acid, siderophore, and terpenoid biosynthesis while dicot-cutter ant fungus gardens metagenomes are enriched in genes involved in membrane transport. Our results suggest that bacteria in leaf-cutter ant fungus gardens aid in nutrient supplementation, a function especially relevant for the fungus gardens of ants that forage grass, a plant source relatively lower in nutrient value.

Introduction

Understanding the role of microbial symbionts in aiding nutrient acquisition is fundamental to understanding the biology of herbivores. Most herbivores host microbial symbionts that serve as an interface between them and the plants that they consume. These microbes can compensate for the hosts' lack of physiological capacity to obtain energy and nutrients from plants (Hansen and Moran, 2013). Herbivore microbial symbionts, often residing in the guts of animals, have been implicated in aiding plant biomass breakdown (Hess *et al.*, 2011; Kudo, 2009; Talbot, 1977; Adams *et al.*, 2011), plant defense compound remediation (Wang *et al.*, 2010; Adams *et al.*, 2013; Boone *et al.*, 2013), and nutrient supplementation (Warnecke *et al.*, 2007; Hansen and Moran, 2011; LeBlanc *et al.*, 2013). Microbial communities differ between hosts that specialize on different substrates (Muegge *et al.*, 2011) and changes in these communities and their functional capacity are integral to their hosts' transition to utilizing novel substrates (Hammer and Bowers, 2015; Delsuc *et al.*, 2013; Li *et al.*, 2015; Kohl *et al.*, 2014; 2016).

Leaf-cutter ants represent a paradigmatic example of the microbial mediation of herbivory. They are dominant herbivores in the Neotropics, consuming up to an estimated 17% of foliar biomass in the systems in which they live (Costa *et al.*, 2008). These ants have a significant impact on their surrounding ecosystems, due to the volume of plant biomass they consume and soil that they excavate in building their underground colonies (Herz *et al.*, 2007; Costa *et al.*, 2008; Fowler *et al.*, 1986; Moutinho *et al.*, 2003; Gutiérrez and Jones, 2006). Like other metazoans, leaf-cutter ants lack the capacity to break down recalcitrant plant material. Instead, they gain access to the nutrients in plant biomass by farming a fungus, *Leucoagaricus gongylophorus*, which serves as an external gut that enzymatically breaks down recalcitrant biomass in the leaf material that the ants forage (Aylward *et al.*, 2013; Khadempour *et al.*,

2016; Suen *et al.*, 2011a; Grell *et al.*, 2013; Kooij *et al.*, 2011; Nagamoto *et al.*, 2011). *Leucoagaricus gongylophorus* produces gongylidia, specialized hyphal swellings that contain an abundance of sugars and lipids, that the ants consume and feed to larvae (Bass and Cherrett, 1995; North *et al.*, 1997).

Recent work has revealed that a community of bacteria reside within leaf-cutter ant fungus gardens (Suen *et al.*, 2010; Aylward *et al.*, 2012; Moreira-Soto *et al.*, 2017). These communities are dominated by Gammaproteobacteria, and consistently contained strains of *Pseudomonas*, *Enterobacter* and either *Rahnella* or *Pantoea,* and are highly similar to communities of bacteria associated with other fungus-farming insects (Aylward *et al.*, 2014). Some garden bacteria are vertically transmitted, maternally through the fungus pellets that alate queens use to establish new fungus gardens (Moreira-Soto *et al.*, 2017). The community consistency and their vertical transmission, suggest that the bacterial communities are important to the fitness of their hosts. One study, by Pinto-Tomás *et al.* (2009) showed that *Pantoea* and *Klebsiella* bacteria fix nitrogen that supplements the ant diet, which is important for a strict herbivorous system. Nevertheless, the functional role of most garden bacteria remains unknown.

While most leaf-cutter ants use dicots, three species of *Atta* are specialized on cutting grass, and another three species cut both grasses and dicots (Fowler *et al.*, 1986). All previous studies on the microbial community in leaf-cutter ant fungus gardens have been focused on dicot-cutting ants, likely because dicot-cutters are more common and grass-cutter ants are notoriously difficult to maintain in the lab (Nagamoto *et al.*, 2009). In this study, we compare the bacterial communities of fungus gardens from ants that cut grass and dicots. Given that grasses and dicots differ in terms of the cell wall composition (Popper and Tuohy, 2010; Ding and Himmel, 2008), plant defense compounds (Wetterer, 1994; Mariaca *et al.*, 1997)

and nutrient availability (Mattson, 1980; Winkler and Herbst, 2004), we hypothesize that the bacterial community in these fungus gardens will differ in terms of community composition and functional capacity, in response to the different composition of the substrates the ants incorporate into their gardens. To address this, we collected fungus gardens from grass- and dicot-cutter ants and obtained their metagenomes using Illumina sequencing. We analyzed the bacterial community in terms of its taxonomic composition and its functional capacity. We also conducted analyses on the fungus gardens to determine their plant composition, their nutritional composition and their plant defense compound contents.

Methods

Collection of fungus garden

Fungus gardens were collected on the campuses of the University of São Paulo (USP) in Ribeirão Preto, SP, Brazil and the São Paulo State University (UNESP) in Botucatu, SP, Brazil. Collection dates and GPS coordinates are listed in Table 1. We collected fungus gardens from four species of *Atta* leaf-cutter ants: *A. bisphaerica* and *A. capiguara*, which are both described as grass-cutters, *A. laevigata*, which is described as a grass and dicot-cutter, and *A. sexdens*, which is described as a dicot-cutter (Fowler *et al.*, 1986).

To collect the fungus gardens, we identified the ant species by worker morphology then followed the entrance tunnel by digging until we found a fungus garden. Care was taken to expose fungus garden chambers from the side, to avoid damaging the garden with digging tools and to avoid contamination with surrounding soil. Fungus gardens were transported to the laboratory and aseptically transferred into 50 mL conical tubes. The majority of worker ants were removed from the fungus garden material before being transferred to the tubes. In order to further reduce the chance of soil contamination, only intact fungus garden from the central region of the fungal mass was included in the tubes. Once filled, the tubes were

frozen in liquid nitrogen and stored at -80°C. At least six 50 mL conical tubes were filled from each colony. For each colony, four tubes were used for metagenomics, one tube was used for gas chromatography, and one tube was used for iron content measurements.

DNA extraction

To target the bacteria in the fungus gardens, DNA was extracted by first using a differential centrifugation method (Aylward *et al.*, 2012). PBS buffer with 1% tween 80 was added to the tubes and they were shaken for 30 min on a vortex. They were then kept at 4°C for 30 min so that large particles would settle. The liquid portion was decanted and passed through a 40 μm filter. The remaining leaf material from the fungus gardens was photographed after the differential centrifugation, to demonstrate the difference in leaf material consistency (Figure 1). The filtrate was centrifuged for 30 min at 4°C, after which a bacterial cell pellet was formed and the liquid was removed. This process was repeated with the original fungus garden tube. For each fungus garden, cell pellets from four tubes were combined and the DNA was extracted using the Qiagen Plant DNA Extraction Maxi Kit (Qiagen, Hilden, Germany).

DNA sequencing and assembly

All metagenomic sequencing was conducted at the Joint Genome Institute in Walnut Creek, CA. Since some of the DNA concentrations were too low for standard library prep, a low-input prep was completed for all of the samples. Sequencing was performed on an Illumina HiSeq-2500 platform (2 x 151 bp). BBDuk adapter trimming (Bushnell, 2017) was used to remove known Illumina adapters. The reads were then processed using BBDuk filtering and trimming. Read ends were trimmed where quality values were less than 12. Read pairs containing more than three ambiguous bases, or with quality scores (before trimming) averaging less than three over the read, or length under 51 bp after trimming, as

143 well as reads matching Illumina artifact, spike-ins or phiX were discarded. Trimmed,
144 screened, paired-end Illumina reads were assembled using the megahit assembler using with
145 the "--k-list 23,43,63,83,103,123" option. Functional annotation and taxonomic classification
146 were performed using the Integrated Microbial Genomes pipeline.

147 *Plant genus richness*

148 To determine the richness of plant substrate integrated in the fungus gardens of the
149 ants, we used JGI's Integrated Microbial Genomes and Microbiomes (IMG) database "find
150 gene" function to retrieve all genes annotated as *MatK* from the dataset. *MatK* is a widely
151 used chloroplast plant DNA barcode (Hollingsworth *et al.*, 2011). Retrieved *MatK* sequences
152 for each metagenome were identified using BLAST. To ensure consistent and reliable
153 certainty with the identified plants, we identified all sequences to the genus level. Because
154 most of the plant biomass was removed from samples before DNA extraction only
155 presence/absence of genera were considered, not abundance.

156 *Bacterial taxonomic analysis*

157 Abundance of bacterial groups (phyla and genera) were determined based on the
158 IMG Phylogenetic Distribution tool, which is part of JGI's standard operating procedure
159 (Huntemann *et al.*, 2016). Briefly, IMG uses USEARCH (Edgar, 2010) to compare
160 metagenome gene sequences to all identified genomes in their database. One top USEARCH
161 hit per gene is used to assign phylogenetic lineage. To determine relative abundance of
162 bacterial taxonomic groups within each sample, we used the PhyloDist raw data from IMG
163 and first removed all gene sequences that were identified as Eukaryote or Virus. We then
164 matched the PhyloDist data to the gene counts for each gene and normalized them to the
165 total number of genes from Bacteria and Archaea (Zhang *et al.*, 2015). We used the relative
166 abundances of each phylum and genus to run an non-metric multidimensional analysis

(NMDS) using a Bray-Curtis dissimilarity index with the vegan package in the R statistical programming environment (Oksanen *et al.*, 2013; R Core Team, 2013). Also using the vegan package, we used ANOSIM and PERMANOVA to determine if groups (grass-cutters vs. dicot-cutters) were significantly different, and we used the Shannon diversity index to compare the diversity of each sample by bacterial genus. To test whether specific genera have significantly different relative abundances between grass- and dicot-cutter ant fungus gardens, we used DESeq2 in the R statistical programming environment (Love *et al.*, 2014). Since DESeq2 requires inputs to be integers, we used number of gene copies per million genes in the metagenomes as our input (Alneberg *et al.* 2014).

Bacterial functional analysis

In order to make functional comparisons of the bacteria in grass- and dicot-cutter fungus gardens, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations of the metagenomes through IMG's KEGG Orthology (KO) pipeline, which is part of JGI's standard operating procedure (Huntemann *et al.*, 2016). Briefly, genes were associated with KO terms (Kanehisa *et al.*, 2014) based on USEARCH 6.0.294 results (Edgar, 2010) and were filtered for minimum identity matches and gene sequence coverage. For an overall comparison of functional differences between the fungus gardens, we used the same ordination and statistical methods as for bacterial genus abundance. As with genus group differences, we used DESeq2 to determine what genes are significantly enriched between grass- and dicot-cutter ant fungus gardens, with number of gene copies per million genes in the metagenomes as our input (Alneberg *et al.* 2014).

Iron content

Separate 50 mL tubes of fungus garden material, from the same colonies as above, were used for determination of iron content. All ants were removed from fungus garden

191 then the remaining material was analyzed at the UW Soil and Forage Lab in Marshfield, WI,
192 using standard methods. Total iron content was determined by first digesting the fungus
193 garden material in nitric acid/peroxide then analyzing by inductively coupled plasma optical
194 emission spectroscopy (ICP-OES) (Fassel and Kniseley, 1974).

195 **Results**

196 *Metagenomic statistics*

197 A summary of metagenome statistics is presented in (Table 2). A total of 5.316 Gbp
198 of assembled sequence data was produced in this study, with an average of 443 Mbp per
199 metagenome. The smallest metagenome was from the grass-cutter colony *A. capiguara* 1 at
200 148.7 Mbp, and the largest metagenome was from the dicot-cutter colony *A. sexdens* 2 at
201 812.9 Mbp. Maximum scaffold lengths ranged from 61.96 Kbp to 701.42 Kbp, with an
202 average maximum scaffold length of 266.6 Kbp. Between 91.63% and 99.31% of reads were
203 aligned.

204 *Bacterial taxonomic analysis*

205 Proteobacteria (70-99%) were the most abundant bacterial phylum detected in the
206 fungus gardens of *Atta* spp., followed by Actinobacteria (0.13-24%) and Firmicutes (0.096-
207 2.4%) (Supplemental Figure 1). Between fungus gardens, genus-level comparisons showed
208 greater variability than phylum-level comparisons (Figure 2, Figure 3). Overall, *Pantoea* was
209 the most abundant genus in all the fungus gardens (average 37%), followed by *Pseudomonas*
210 (average 17%). The abundance of these two genera was especially pronounced in the grass-
211 cutter ant fungus gardens, where *Pantoea* and *Pseudomonas* averaged 45% and 28%,
212 respectively. The high relative abundance of these two genera contributed to a lower overall
213 diversity in the grass-cutter ant gardens (Shannon diversity index of 1.20-2.44) (Figure 3).
214 While *Pantoea* and *Pseudomonas* were still abundant in fungus gardens of the dicot-cutter ants,

215 *A. laevigata* and *A. sexdens*, it accounted for a lower proportion (28% and 6.5%, respectively)
216 of the bacteria in these more diverse gardens (Shannon diversity index of 2.80-4.67). Other
217 dominant bacterial genera included *Enterobacter, Burkholderia, Erwinia, Emticicia, Serratia* and
218 *Klebsiella*. DESeq2 analysis revealed that six bacterial genera (*Entoplasma*, *Flavobacterium*,
219 *Mesoplasma*, *Pantoea*, *Pseudomonas*, and *Spiroplasma*) were significantly different in relative
220 abundance between the fungus gardens. They were all more abundant in the grass-cutter ant
221 fungus gardens.

222 *Bacterial functional analysis*

223 Overall, we found significant differences in the predicted bacterial community
224 functional profiles between grass- and dicot-cutter ant fungus gardens (Figure 4). All
225 individual bacterial genes that were significantly different between grass- and dicot-cutter ant
226 fungus gardens are listed in Supplemental Table 1. In total, 514 predicted bacterial genes
227 were significantly enriched, with 313 and 201 genes significantly enriched in grass- and dicot-
228 cutter ant gardens, respectively (Supplemental Table 2, Supplemental Figures 4-6). Grass-
229 cutter ant fungus gardens were enriched for amino acid biosynthesis genes for phenylalanine,
230 tryptophan, tyrosine, histidine, arginine, lysine, cysteine, methionine, glycine, serine and
231 threonine. They were also significantly enriched in terpenoid and siderophore biosynthesis
232 genes (Figure 5) and had a significantly higher abundance of a gene in the nitrogen fixation
233 pathway, nitrogenase molybdenum-iron protein beta chain (Supplementary Table 2). Dicot-
234 cutter ant fungus gardens were particularly enriched in membrane transport genes (Figure 5).

235 *Plant taxonomy and consistency*

236 The incorporated plant material was markedly different in consistency between the
237 fungus gardens. *Atta bisphaerica* and *A. capiguara* gardens both contained material that was
238 clearly grass, which was not mulched (Figure 1). In contrast, the leaf material in the fungus

239 gardens of *A. laevigata* and *A. sexdens* was mulched to the point of being unrecognizable as
240 plant material (Figure 1). We detected 68 plant species based on the *MatK* gene query in the
241 metagenomes, from 40 genera and 15 families (Table 3). The fungus gardens of dicot-cutter
242 ants had a significantly higher richness of plant genera than those of grass-cutter ants
243 (ANOVA F=9.14, p=0.0128). As expected, the grass-cutter ant fungus gardens all contained
244 grass (*Paspalum*, Poaceae). The dicot-cutter ant fungus gardens contained more genera and
245 families of plants, which were mostly dicots, but three of these fungus gardens also
246 contained some grass (Table 3).

247 *Iron content*

248 The iron content of the fungus gardens is displayed in Figure 6. The grass-cutter ant
249 fungus gardens have lower amounts of iron than the dicot-cutter ant fungus, but this
250 difference is not significant due to the high variability between *A. sexdens* gardens.

251 **Discussion**

252 Understanding how microbial symbiont communities change in relation to host
253 substrate specialization can help inform on animal diet specialization and evolutionary
254 transitions to utilizing novel substrates. *Atta* ants provide a relatively unique opportunity to
255 examine a group of closely related herbivores that have transitioned from specialization on
256 dicots to grasses. Dicots and grasses differ in terms of their cell wall composition, nutrient
257 content and plant defense compounds. Here, using metagenomic sequencing, we examine
258 this transition in the bacterial community in the fungus gardens of ants that are specialized
259 on these different substrates. The results of this study demonstrate that the bacterial
260 community differs depending on type of substrate and likely facilitates the ants' ability to
261 specialize on grasses, which represents a lower quality of substrate on which to grow their
262 fungal crop.

263 If bacteria in fungus gardens are responsible for the breakdown of recalcitrant plant
264 biomass, which is found in plant cell walls, we expect that the bacterial communities in the
265 two ant groups examined here would be differentially enriched in the genes necessary for
266 plant biomass breakdown. Grasses have a unique cell wall structure, containing
267 (1→3),(1→4)-β-D-glucan chains and silica, neither of which are present in dicots (Popper
268 and Tuohy, 2010). In other systems specialized on grass biomass breakdown, the microbes
269 responsible for this produce specialized enzymes (King *et al.*, 2011) and have genomes that
270 are adapted for this function (Wolfe *et al.*, 2012). We do not observe any changes in
271 abundance of plant biomass degrading genes between the two systems. Thus, we can
272 conclude that garden bacteria do not respond to changes in cell wall structure between
273 grasses and dicots. Instead, it is likely that the genome or gene expression in the fungus from
274 these two systems would show differences, especially since the fungus is the primary
275 degrader of plant biomass in leaf-cutter ant fungus gardens (Aylward *et al.*, 2013;
276 Khadempour *et al.*, 2016; Nagamoto *et al.*, 2011; Grell *et al.*, 2013).
277 Leaf-cutter ants, in general, cut an exceptionally broad diversity of plants (Solomon,
278 2007; Mayhé-Nunes and Jaffe, 1998) and thus, have the potential to encounter a myriad of
279 plant defense compounds that are toxic to themselves and their fungal cultivar. The ants are
280 not enriched in genes families for plant defense compound detoxification (Rane *et al.*, 2016),
281 so they must reduce the intake of these chemicals in other ways. Plant defense compound
282 avoidance occurs in several steps. First, ants avoid cutting plants that contain plant defense
283 compounds that are particularly toxic or abundant (Wirth *et al.*, 1997; Hubbell *et al.*, 1984;
284 Howard, 1988). Second, many plant defense compounds that the ants encounter are volatile
285 chemicals (Howard, 1988; Howard *et al.*, 1988), and in the time that the ants cut and carry
286 the leaf material back to their colonies, some of the volatiles will have had time to dissipate.

287 Finally, ants often leave leaf material in caches before they incorporate them into their
288 fungus gardens (Hart and Ratnieks, 2000; Roschard and Roces, 2003), providing further
289 opportunity for the defense compounds to evaporate. Nevertheless, some amount of
290 volatiles can make their way into the gardens. In this study, using gas chromatography, we
291 were able to detect eucalyptus-related compounds (eucalyptol, α-pinene, β□ pinene, *p*-
292 cymene and γ-terpinene) in the fungus garden of one ant colony (*A. laevigata* 1) that was
293 observed cutting considerable amounts of eucalyptus (Supplemental methods and
294 Supplemental Figure 2).

295 In order to mitigate the deleterious effects of plant defense compounds, we expect
296 the fungal cultivar *L. gongylophorus* would produce enzymes to degrade them. Indeed, work by
297 De Fine Licht *et al.* (2013) implicates an important role for laccases from the fungal cultivar
298 in detoxifying plant defense compounds. Nevertheless, bacteria in the garden may also play a
299 role in mediating plant defense compounds. The bacterial community contains the genes
300 necessary for plant defense compound remediation, including many cytochrome P450s,
301 gluthione S-transferases, and other genes involved in xenobiotic degradation, and aromatic
302 compound degradation, but they are not consistently enriched in the dicot-cutter ant fungus
303 gardens (Supplemental Table 1). We expected that since dicot-cutter ants incorporate a
304 higher diversity of plants into their gardens (Table 3), that the diversity of bacteria would
305 also be higher in these gardens, and that the bacteria would have a higher capacity for the
306 degradation of these defense compounds. While we did observe a greater diversity of
307 bacteria in the dicot-cutter ant fungus gardens (Figure 3) we did not see a significant
308 enrichment of plant defense compound degradation genes in these gardens (Figure 5,
309 Supplemental Table 1). However, we still cannot exclude the possibility that bacteria are
310 taking part in this process. Since each dicot-cutter ant colony cuts a unique set of plants

311 (Table 3), they potentially encounter a unique set of plant defense compounds. If the
312 bacterial community were to respond in a substrate-specific manner to different plant
313 defense compounds, our analysis in this study would not reveal that. To elucidate the role of
314 bacteria in plant defense compound remediation, closely controlled experiments with
315 particular defense compounds of interest applied to bacterial cultures and to fungus gardens
316 would be necessary.

317 Pinto-Tomas *et al.* (2009) established that *Pantoea* and *Klebsiella* bacteria in Central
318 American leaf-cutter ant fungus gardens are supplementing the ant diet through nitrogen
319 fixation. Plant material, in general, is low in nitrogen, and many herbivores supplement their
320 diets through bacterial nitrogen fixation (Douglas, 2009; Hansen and Moran, 2013). Grasses
321 are especially low in nitrogen (Mattson, 1980; Winkler and Herbst, 2004), so we predict that
322 grass-cutter ant fungus gardens would be enriched in nitrogen-fixing bacteria with a
323 corresponding enrichment of nitrogen-fixing genes. Here we show that *Pantoea* are more
324 abundant in the grass-cutter ant fungus gardens, and that a nitrogenase molybdenum-iron
325 protein beta chain gene is significantly more abundant in grass-cutter ant fungus gardens
326 (Supplemental Table 1). Other genes that are related to nutrient acquisition are also
327 significantly more abundant in the grass-cutter ant fungus gardens (Figure 5), such as genes
328 in amino acid metabolism pathways. While it has been shown that nitrogen fixed by bacteria
329 is incorporated into the bodies of ants (Pinto-Tomás *et al.*, 2009), animals cannot simply
330 absorb nitrogen as ammonium or nitrate, they require it to either be in the form of amino
331 acids or other organic nitrogen-containing compounds (White, 1993). The enrichment of
332 arginine biosynthesis genes is of particular interest since the genome of *Atta* is deficient in
333 genes in this pathway (Suen *et al.*, 2011b). While a transcriptome study of *L. gongylophorus*

334 demonstrated that the cultivar has the genes necessary for arginine biosynthesis (De Fine
335 Licht *et al.*, 2014), the bacteria could supplement this process.

336 Other categories of genes enriched in the grass-cutter ant fungus garden bacteria are
337 those involved in metabolism of terpenoids and other secondary metabolites, especially their
338 biosynthesis. Grass-cutter ant fungus gardens are significantly enriched in 67 of these genes.
339 This list includes seven siderophores, which are responsible for iron acquisition (Crosa,
340 1989; Winkelmann, 2002). Siderophores are costly to produce so the enrichment of these
341 genes suggests that iron acquisition is important in this system. The grass-cutter ant fungus
342 gardens examined in this study contained lower amounts of iron than the dicot-cutter ant
343 fungus gardens (Figure 6). Terpenoids are the most abundant secondary metabolites found
344 in plants, and serve diverse roles (Langenheim, 1994; Gershenzon and Dudareva, 2007). The
345 majority of research into the connection between plant terpenoids and animal-microbe
346 symbioses are in regards to the detoxification of terpenes that would be deleterious to the
347 animal host (Cheng *et al.*, 2013; Raffa, 2013; Adams *et al.*, 2013; Boone *et al.*, 2013; Wang *et*
348 *al.*, 2012). However, not all terpenes are toxic to all organisms (Raffa, 2013), and in at least
349 one instance they have been shown to supplement a herbivore's diet after some modification
350 by a gut bacterium (Berasategui *et al.*, 2017). Dicots contain higher quantities of terpenoids
351 (Wetterer, 1994; Mariaca *et al.*, 1997). One possibility is that the bacteria in these fungus
352 gardens are producing terpenes as a nutritional additive, especially in the grass-cutter ant
353 fungus gardens where there are lower terpene inputs and these genes are enriched (Figure 5,
354 Supplemental Figure 2).

355 Grass-cutter ants are adapted to cutting grass; with workers that have shorter, wider
356 mandibles than their dicot-cutter counterparts, which may facilitate slicing grass blades (Silva
357 *et al.*, 2016; Fowler *et al.*, 1986). They also process leaves differently – they do not mulch the

358 material, likely because the silica contained in grasses would dull their mandibles (Silva *et al.*,
359 2016; Massey and Hartley, 2009). The ants' adaptation to grass-cutting, combined with the
360 apparent specialized bacterial community in the gardens, allow grass-cutter ants to use grass
361 as a substrate more efficiently than dicot-cutter ants can. This has allowed grass-cutter ants
362 to exploit a novel niche, presumably reducing the amount of interspecific competition they
363 experience.

364 Optimal foraging theory predicts that when the quality of forage is lower, leaf-cutter
365 ants should be cutting a greater diversity of plants (Rockwood and Hubbell, 1987). While
366 grass-cutter ants forage lower-quality material than their dicot-cutter counterparts, they also
367 cut a significantly lower diversity of plants. This is because grass-cutter ants to do not have
368 access to the diversity of plants necessary to compensate for their low forage quality. Instead,
369 the bacteria in their fungus gardens may be providing the necessary nutrition that a diverse
370 diet provides in dicot-cutter ant fungus gardens, allowing grass-cutter ant species to exploit
371 this novel niche. Our work builds on previous studies that provide evidence for the
372 mutualistic role of bacteria in leaf-cutter ant fungus gardens and it furthers the idea that
373 microbial symbionts are important players in novel substrate utilization by animals.
374

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388 Table 1 Summary of collection details for leaf-cutter ant colonies used in this study

Leaf-cutter ant colony	Substrate niche	IMG genome number	Collection date	Latitude	Longitude
A. bisphaerica 1	Grass	3300013023	1-Feb-15	S22°50'47.7"	W48°26'.9"
A. bisphaerica 2	Grass	3300013025	3-Feb-15	S22°50'48.4"	W48°26'1.4"
A. bisphaerica 3	Grass	3300013022	3-Feb-15	S22°50'48.4"	W48°26'2.3"
A. capiguara 1	Grass	3300012994	2-Feb-15	S22°54'32.1"	W48°18'28.7"
A. capiguara 2	Grass	3300012996	3-Feb-15	S22°50'47.2"	W48°26'1.3"
A. capiguara 3	Grass	3300012997	3-Feb-15	S22°50'47.6"	W48°26'1.2"
A. laevigata 1	Dicot*	3300013000	20-Jan-15	S21°9'55.5"	W47°50'51.3"
A. laevigata 2	Dicot*	3300012995	17-Jan-15	S21°10'3"	W47°50'47"
A. laevigata 3	Dicot*	3300012998	19-Jan-15	S21°9'56.8"	W47°50'52.7"
A. sexdens 1	Dicot	3300012999	30-Jan-15	S21°9'50"	W47°51'26.9"
A. sexdens 2	Dicot	3300013002	30-Jan-15	S21°9'53.4"	W47°51'10.5"
A. sexdens 3	Dicot	3300013001	31-Jan-15	S21°10'2"	W47°51'5"

389 *While *A. laevigata* has been described as a grass/dicot-cutter ant (Fowler *et al.*, 1986), due to its leaf-processing
390 behavior and fungus garden plant composition observed in this study, we consider it a dicot-cutter.
391

392 Table 2 Metagenome sequencing statistics for leaf-cutter ant fungus gardens

Leaf-cutter ant colony	Scaffold total	Scaffold sequence total (Mbp)	Main genome scaffold N/L50	Main genome scaffold N/L90	Max scaffold length (Kbp)	Scaffolds > 50 Kbp	Aligned reads	Protein coding genes
A. bisphaerica 1	628724	390.8	122506/ 740	467228/ 298	249.52	93 (2.07%)	163185122 (98.76%)	607 042 (99.39%)
A. bisphaerica 2	939707	630.9	148370/ 926	680177/ 292	253.54	69 (0.88%)	148406252 (96.49%)	910 609 (99.61%)
A. bisphaerica 3	285649	186.0	29401/ 972	244722/ 247	187.50	49 (1.90%)	167169016 (98.76%)	358 547 (98.19%)
A. capiguara 1	205334	148.7	16608/ 1403	178745/ 247	273.83	37 (2.49%)	199009346 (99.31%)	272 096 (98.99%)
A. capiguara 2	345332	261.2	35330/ 1420	303130/ 247	180.27	34 (1.06%)	204772230 (98.31%)	456 916 (98.70%)
A. capiguara 3	573737	359.5	83958/ 790	508567/ 247	135.51	13 (0.29%)	203079026 (98.77%)	644 865 (98.79%)
A. laevigata 1	853367	517.5	178678/ 686	645038/ 301	274.98	87 (1.53%)	178461364 (96.65%)	871 330 (99.42%)
A. laevigata 2	295365	189.2	32897/ 990	266928/ 247	252.44	92 (4.23%)	189659024 (96.85%)	332 737 (96.95%)
A. laevigata 3	601593	546.3	74824/ 1744	398992/ 340	241.71	17 (0.30%)	209535750 (96.06%)	722 718 (99.01%)
A. sexdens 1	674609	708.4	48220/ 3118	412923/ 341	701.43	167 (2.40%)	156148622 (97.92%)	822 403 (99.46%)
A. sexdens 2	857038	812.9	65552/ 2346	548662/ 328	61.96	17 (0.11%)	150809208 (95.32%)	1 088 719 (99.51%)
A. sexdens 3	1006806	564.7	221493/ 614	772141/ 277	386.55	68 (1.17%)	186976430 (91.63%)	1 029 784 (99.20%)

393

394 Table 3 Plant genera detected in each fungus garden sample using *MatK* gene

Sample	Family	Genus	Species *MatK* match %
A. bisphaerica 1	Fabaceae	*Chamaecrista*	99.3
	Poaceae	*Paspalum*	99.6
	Polygalaceae	*Polygala*	99.3
A. bisphaerica 2	Poaceae	*Paspalum*	99.4
A. bisphaerica 3	Fabaceae	*Chamaecrista*	99.3
	Fabaceae	*Zornia*	100
	Poaceae	*Paspalum*	99.5
	Polygalaceae	*Polygala*	99.0
A. capiguara 1	Poaceae	*Paspalum*	99.7
A. capiguara 2	Poaceae	*Paspalum*	99.6
A. capiguara 3	Fabaceae	*Chamaecrista*	99.3
	Poaceae	*Paspalum*	99.6
A. laevigata 1	Fabaceae	*Pterogyne*	99.4
	Myrtaceae	*Eucalyptus*	99.9
	Poaceae	*Paspalum*	99.5
	Poaceae	*Urochloa*	100
A. laevigata 2	Asteraceae	*Rensonia*	99.6
	Fabaceae	*Centrolobium*	98.2
	Fabaceae	*Schizolobium*	100
A. laevigata 3	Anacardiaceae	*Pachycormus*	98.6
	Asteraceae	*Kingianthus*	95.4
	Asteraceae	*Podanthus*	99.5
	Fabaceae	*Desmodium*	99.8
	Fabaceae	*Lencaena*	100
	Myrtaceae	*Eucalyptus*	99.8
	Poaceae	*Paspalum*	99.9
A. sexdens 1	Anacardiaceae	*Loxopterygium*	98.4
	Asteraceae	*Cymophora*	98.5
	Bignoniaceae	*Tabebuia*	98.1
	Fabaceae	*Andira*	98.6
	Fabaceae	*Batesia*	98.8
	Fabaceae	*Bussea*	100
	Fabaceae	*Libidibia*	99.8
	Fabaceae	*Pterogyne*	100
	Fabaceae	*Tipuana*	99.9
	Malvaceae	*Pachira*	100
	Myrtaceae	*Eucalyptus*	99.6
	Myrtaceae	*Eugenia*	99.8
	Poaceae	*Scutachne*	98.4
	Rubiaceae	*Genipa*	99.2
	Solanaceae	*Lycianthes*	100
A. sexdens 2	Bignoniaceae	*Tabebuia*	98.3
	Combretaceae	*Lumnitzera*	93.4
	Fabaceae	*Centrolobium*	98.2
	Fabaceae	*Pterogyne*	99.3
	Fabaceae	*Tipuana*	100
	Lecythidaceae	*Careya*	94.0
	Santalaceae	*Phoradendron*	99.6
A. sexdens 3	Asteraceae	*Echinacea*	99.3
	Asteraceae	*Eclipta*	100
	Asteraceae	*Perymeniopsis*	99.8
	Asteraceae	*Synedrella*	100
	Commelinaceae	*Commelina*	100
	Commelinaceae	*Murdannia*	92.0
	Fabaceae	*Desmodium*	99.8
	Fabaceae	*Lencaena*	100
	Malvaceae	*Sida*	99.7
	Myrtaceae	*Eucalyptus*	98.6
	Phyllanthaceae	*Phyllanthus*	100
	Rubiaceae	*Genipa*	100
	Solanaceae	*Acnistus*	99.4

395

Figure captions

Figure 1 Grass- and dicot-cutter ants differ in the niches that they occupy, and the way that they cut and process leaf material. Field sites in (A) Botucatu, SP and (B) Ribeirão Preto, SP, Brazil. Fungus gardens of (C) grass- and (D) dicot-cutter ants. C. Visual inspection of leaf material from leaf-cutter ant fungus gardens demonstrates the degree of mulching that the different ants complete, with grass-cutters leaving the leaf material more intact (E – *A. bisphaerica* and F – *A. capiguara*), while dicot-cutters mulch to the point of unrecognizable leaf fragments (G – *A. laevigata* and H – *A. sexdens*).

Figure 2 NMDS plot of the relative abundance of bacterial genera in fungus gardens of grass- and dicot-cutter ants. Grass- and dicot-cutter fungus garden bacterial communities are significantly different.

Figure 3 Genus-level bacterial community analysis of leaf-cutter ant fungus gardens from grass- and dicot-cutter ants, demonstrating that dicot-cutter ant fungus gardens have a higher diversity of bacteria. A. Pie charts showing proportions of different bacterial genera in the fungus gardens. B. Shannon diversity index of bacterial genera. C. Bacterial genus richness (for genera that consist of more than 1% of the total normalize gene count).

Figure 4 NMDS plot of KO functional genes from grass- and dicot-cutter ant fungus gardens. The KO profiles are significantly different between the fungus gardens of ants cutting the different substrates.

Figure 5 Particular groups of genes are enriched in either the grass- or dicot-cutter ant fungus gardens. Grass-cutter ant fungus gardens are enriched for genes involved in metabolism of terpenoids and other secondary metabolites, as well as genes involved in amino acid metabolism. In contrast, dicot-cutter ant fungus gardens are enriched for genes involved in membrane transport. Bars extending to the left (blue) represent genes that are significantly more abundant in dicot-cutter ant fungus gardens and bars extending to the right (red) represent genes that are significantly more abundant in grass-cutter ant fungus gardens.

Figure 6 Iron content of fungus gardens from this study as measured by inductively coupled plasma optical emission spectroscopy. The iron content in the grass-cutter ant fungus gardens was lower than in the dicot-cutter ant fungus gardens. This difference is not statistically significant, however, since the *A. sexdens* fungus garden iron content is highly variable.

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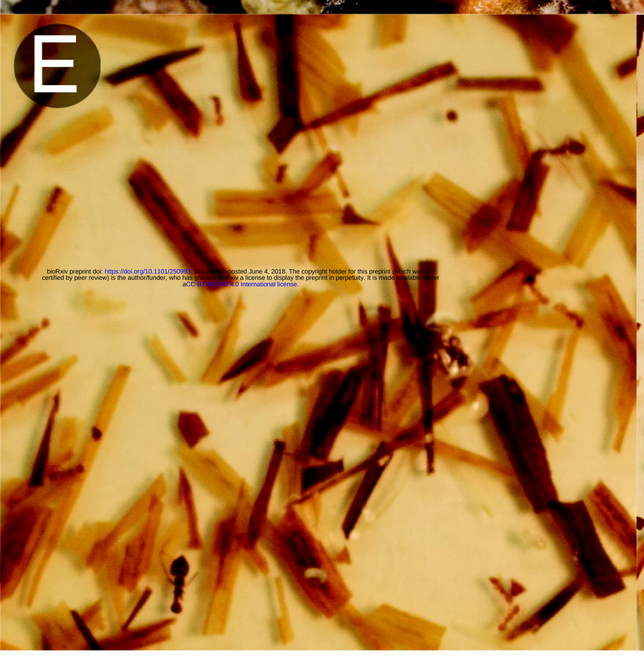
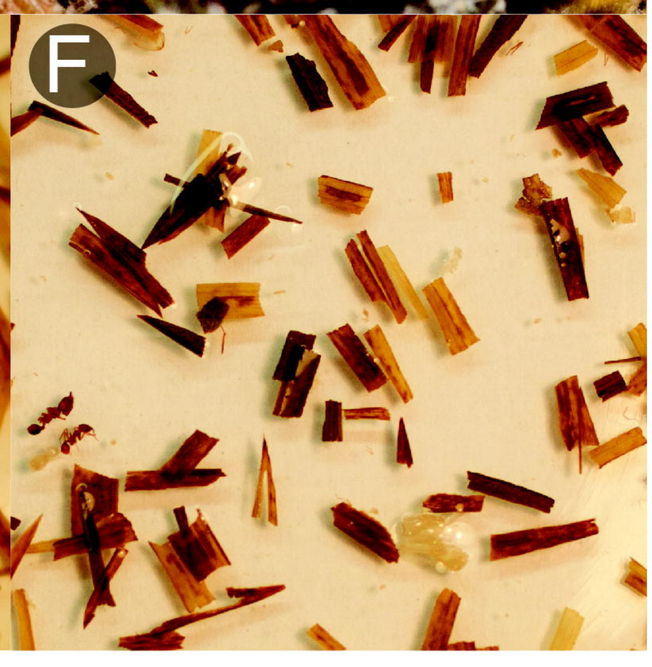
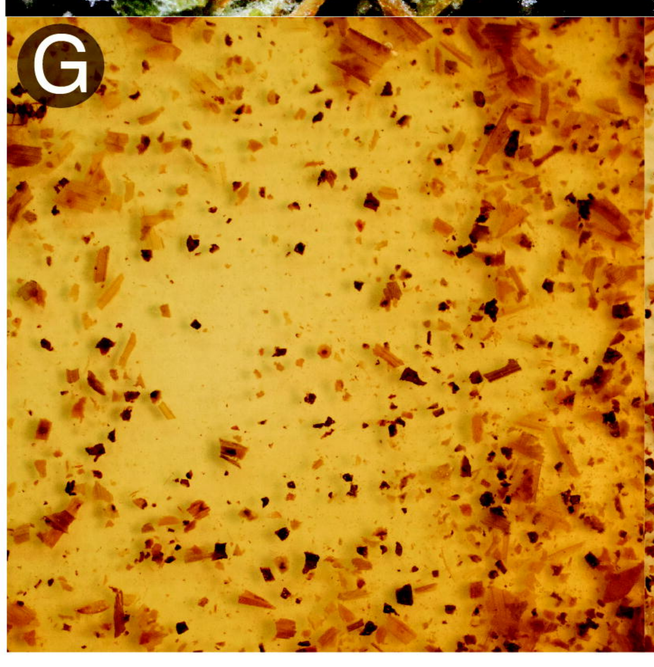
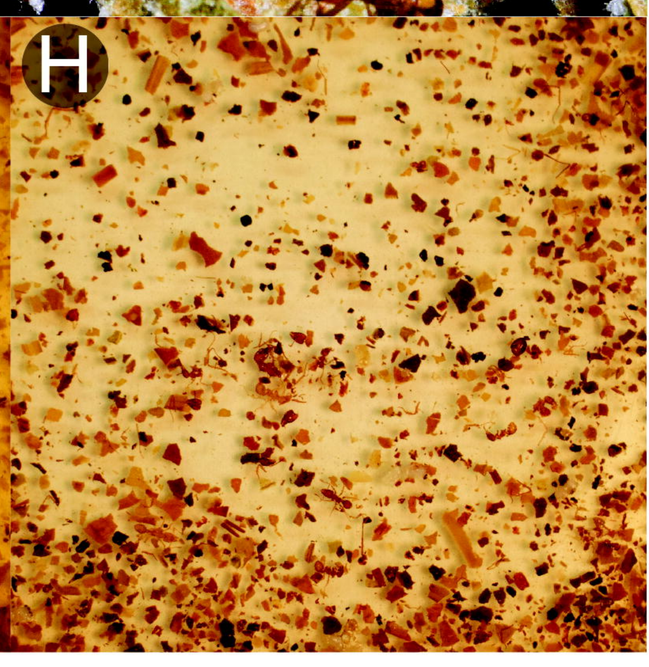


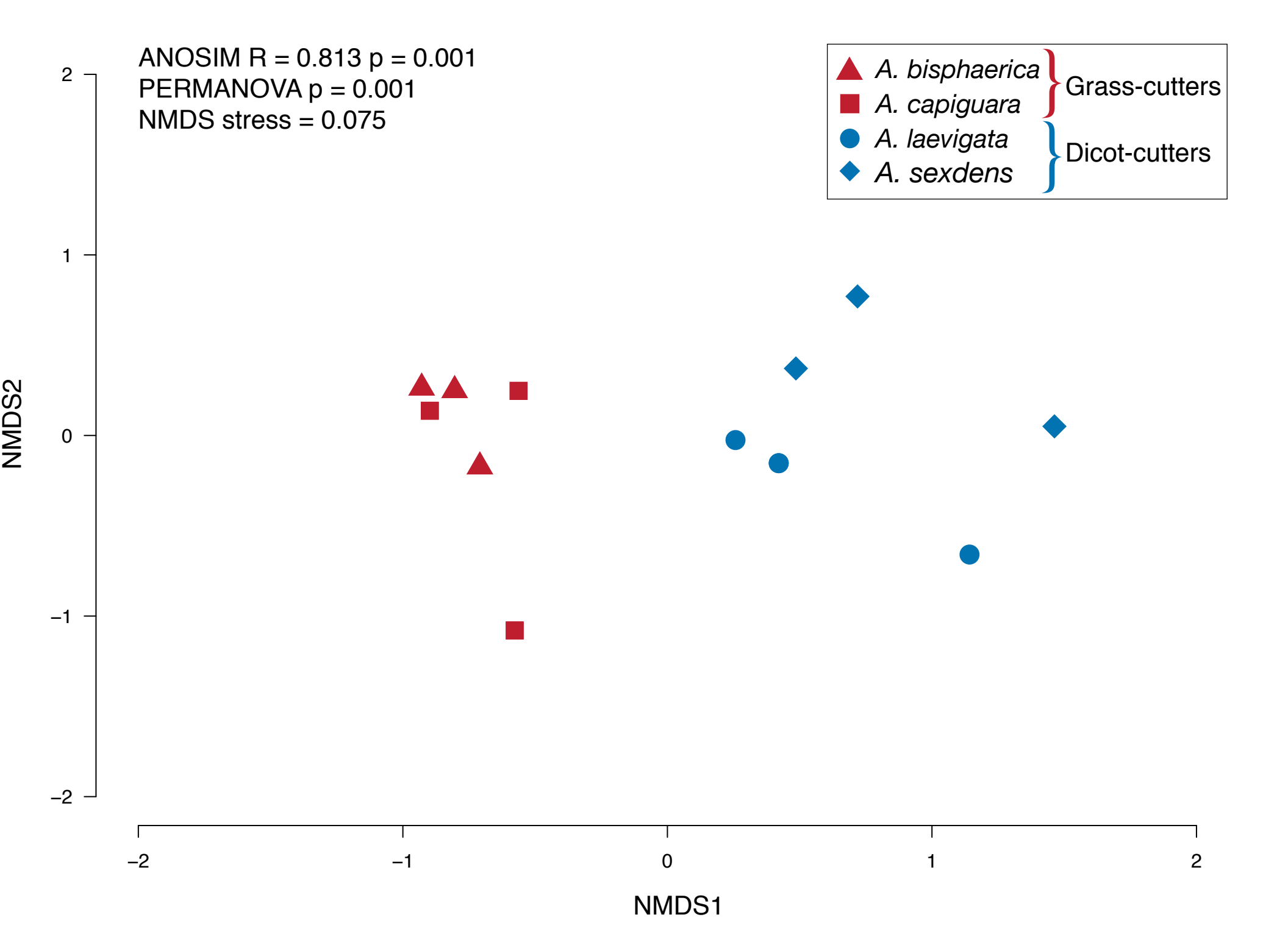


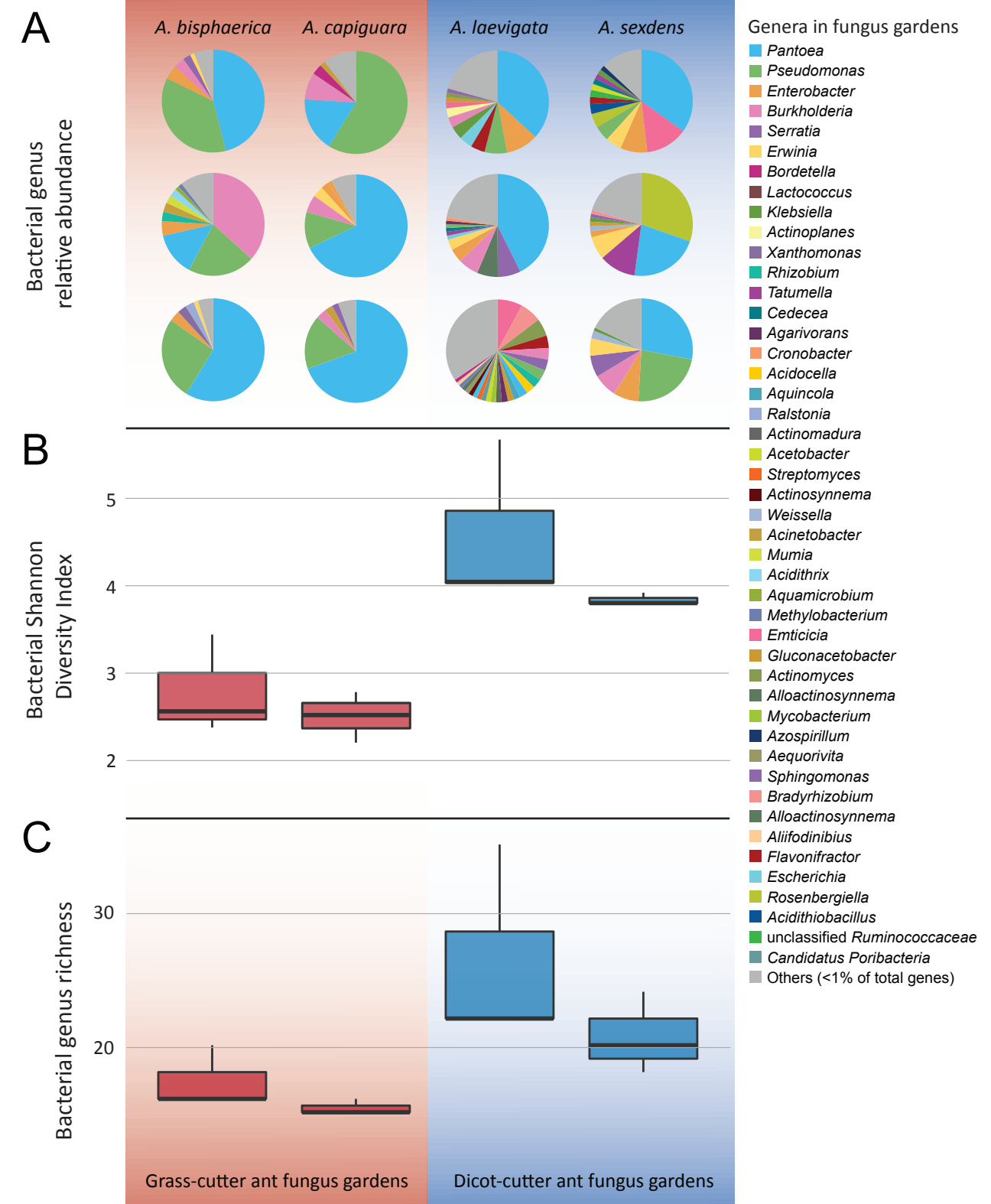


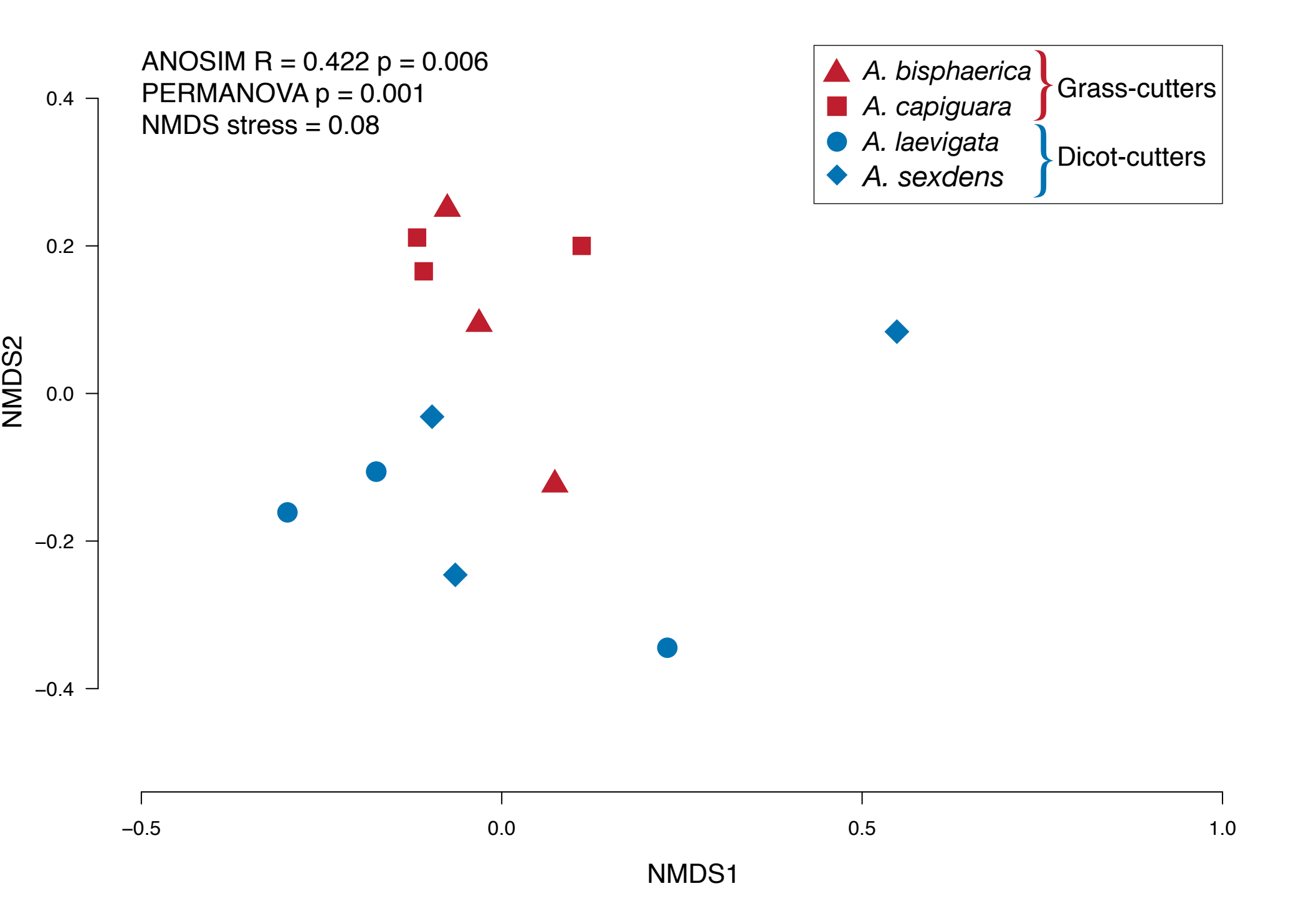


Grass-cutters

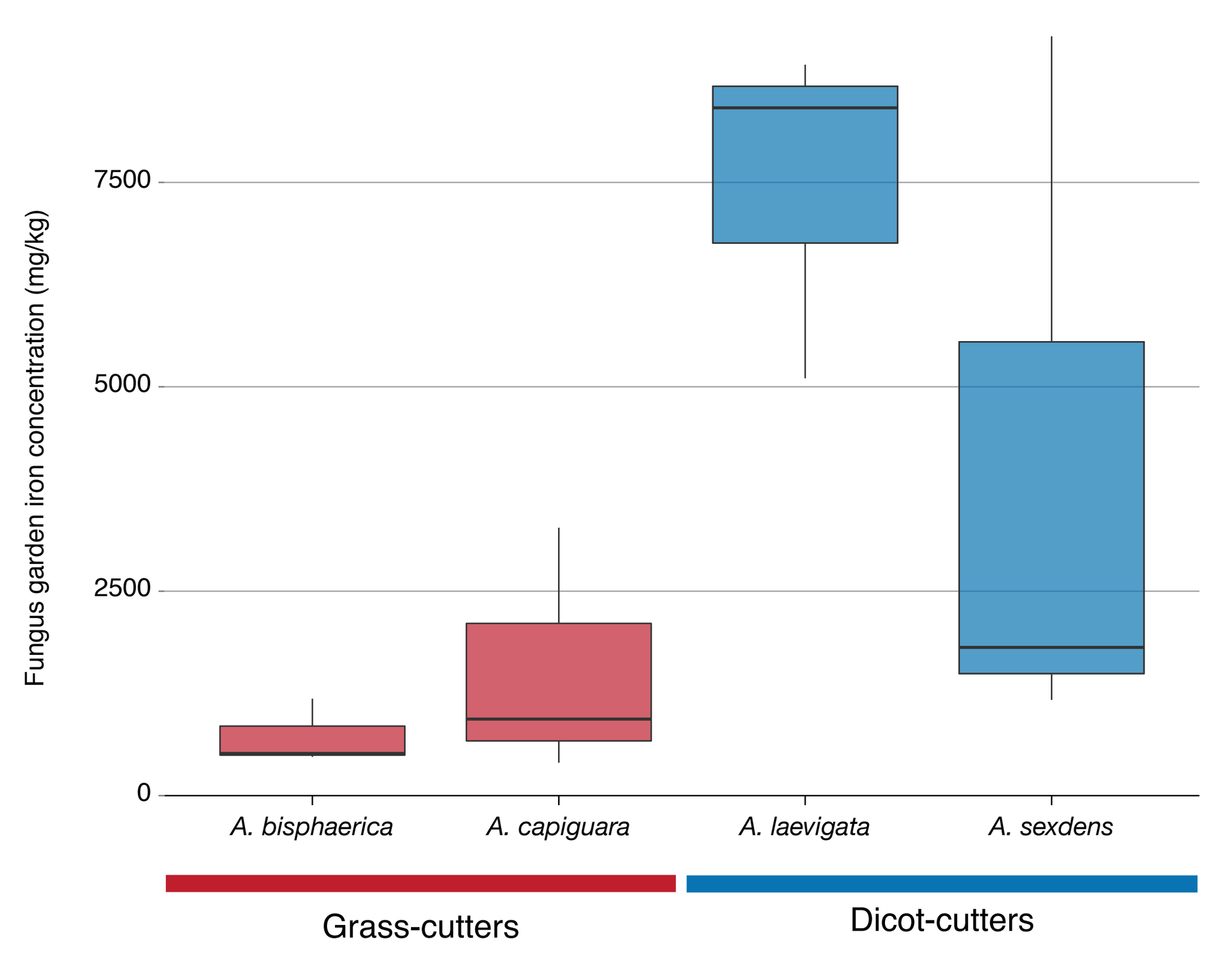
Dicot-cutters


ANOSIM R = 0.813 p = 0.001
PERMANOVA p = 0.001
NMDS stress = 0.075
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Grass-cutters
Dicot-cutters
NMDS1
NMDS2
2
1
0
−1
−2
−2
−1
0
1
2


A
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Bacterial genus relative abundance
B
Bacterial Shannon Diversity Index
5
4
3
2
C
Bacterial genus richness
30
20
Grass-cutter ant fungus gardens
Dicot-cutter ant fungus gardens
Genera in fungus gardens
Pantoea
Pseudomonas
Enterobacter
Burkholderia
Serratia
Erwinia
Bordetella
Lactococcus
Klebsiella
Actinoplanes
Xanthomonas
Rhizobium
Tatumella
Cedecea
Agarivorans
Cronobacter
Acidocella
Aquincola
Ralstonia
Actinomadura
Acetobacter
Streptomyces
Actinosynnema
Weissella
Acinetobacter
Mumia
Acidithrix
Aquamicrobium
Methylobacterium
Emticicia
Gluconacetobacter
Actinomyces
Alloactinosynnema
Mycobacterium
Azospirillum
Aequorivita
Sphingomonas
Bradyrhizobium
Alloactinosynnema
Aliifodinibius
Flavonifractor
Escherichia
Rosenbergiella
Acidithiobacillus
unclassified Ruminococcaceae
Candidatus Poribacteria
Others (<1% of total genes)


ANOSIM R = 0.422 p = 0.006
PERMANOVA p = 0.001
NMDS stress = 0.08
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Grass-cutters
Dicot-cutters
0.4
0.2
0.0
−0.2
−0.4
NMDS2
−0.5
0.0
0.5
1.0
NMDS1


Metabolism of terpenoids and secondary metabolites
Membrane transport
Amino acid metabolism
Biosynthesis of type II polyketide
Biosynthesis of vancomycin group
Nonribosomal peptide structures
Biosynthesis of siderophore group
Type I polyketide structures
Biosynthesis of ansamycins
Sesquiterpenoid and triterpenoid
Zeatin biosynthesis
Carotenoid biosynthesis
Brassinosteroid biosynthesis
Diterpenoid biosynthesis
Terpenoid backbone biosynthesis
Polyketide sugar unit biosynthesis
Biosynthesis of 12-, 14- and 16-membered macrolites
Geraniol degradation
Bacterial secretion system
Phosphotransferase system
ABC transporters
Saccharide, polyol, and lipid transporters
Metalic cation, iron-siderophore and vitamin B12
ABC-2 type and other transporters
Phenylalanine tyrosine and tryptophan metabolism
Tryptophan metabolism
Tyrosine metabolism
Histidine metabolism
Arginine and proline metabolism
Lysine degradation
Cysteine and methionine metabolism
Glycine, serine and threonine metabolism
K02768 tcmO
K02770 elmD
K18893 cepH, sgcC3, mdpC3, kedY3
K18890 dpgD
K17316 bacC
K17240 mxcG
K17238 pchF
K17237 pchE
K17239 pchG
K17215 mbtC
K17214 mbtE
K17213 mbtB
K16299 amphL, nysL, pimD
K15576 rifM, asm45
K15577 rifN, asm22
K15578 rifH
K15554 rifL, asm44
K15553 rifE
K15555 rifK, asm24, asm43
K15496 rifA
K12368 BS, TPS11
K11960 HVS
K11961 TPS21
K11962 CYP71D55
K11963 TPS6_11
K11959 TPS1
K12371 MXPSS1
K12370 EAS
K12369 GERD
K12372 LUP4
K11605 IPT
K11606 UGT76C1_2
K11604 CYP735A
K11607 CISZOG
K10552 ZDS, crtQ
K11073 K10212, crtO
K11075 AOG
K11074 CCS1
K11076 crtX
K11072 crtQ
K10559 crtISO, crtH
K10562 VDE, NPQ1
K10561 ZEP, ABA1
K10545 CYP90D2, D2
K10544 CYP90A1, CPD
K10543 CYP90B1, DWF4
K10539 CPS4
K10538 MAS
K10537 E4.2.3.8
K10117 FOHSDR
K10109 GPS
K10017 hexPS, COQ1
K10015 gcpE, ispG
K10118 FLDH
K09970 dxr
K10014 ispB
K10009 dxs
K10016 ispH, lytB
K09972 FDPS
K10010 ispF
K09815 evaD, eryBVII, aveBV, megDIV, staE
K09816 wbiB
K09817 tll
K06160 tylI, CYP113B
K06858 megY
K05846 eryA
K05847 eryF, CYP107A
K05845 geoB
K05816 atuE
K05813 atuH
K05814 atuF
K05815 atuC
K09523 hlyD, cyaD
K11315 fha1
K03062 impK, ompA, vasF, dotU
K10389 vgrG
K00463 secE
K07326 PTS-Gfr-EIID, gfrD
K12096 PTS-EIIB, sorB-Sor
K12266 PTS-HPR.PTSO, ptsO, npr
K11294 PTS-EIIC, sorA-Sor
K12264 PTS-EI.PTSI, ptsI
K02841 yejB
K03814 ecfA2
K02843 yejE
K19302 iatP
K05286 aguE
K01230 aglG, ggtD
K10661 hisQ
K14026 togB
K10590 ABCA4
K10592 ABCA7
K12757 oleC4
K11090 dasB
K05291 chiG
K05292 gtsA, glcE
K09518 aapJ, bztA
K03850 rbsA
K02739 proV
K09540 modA
K07151 araH
K09580 artQ
K03107 ABC.FEV.S
K01191 alsA
K03361 thiQ
K02735 livF
K00729 ABC.NGC.S
K08496 lptB
K02734 livG
K10967 potI
K02737 livM
K14005 ABC 2.CPSE.P1
K18134 attA1
K02068 ABC.MS.P
K02069 ABC.MS.P1
K02029 ABC.X2.A
K02030 ABC.X2.P
K01992 qrtT
K06148 ABC-2.P
K01990 ABC.CD.P
K01667 quiA
K18383 YUCCA
K02614 ASMT
K05603 E4.1.99.2
K01745 FAHD1
K07008 nagL
K01712 DBH
K18911 hpaI, hpcH
K12256 egtC
K12254 hutH, HAL
K12252 hdc, HDC
K01479 CARNMT1
K12255 hutF
K00673 E4.1.1.19
K09471 spuC
K05526 aruH
K06447 aruI
K10536 L3HYPDH
K01484 puuA
K01585 puuD
K00819 adiA
K01474 astD
K01473 astE
K01470 puo
K03894 SETD2, SET2
K00471 MLL5
K03896 SUV420H
K03895 SETD8
K11419 kdd
K11423 AGPHD1
K05396 DEP1
K00302 GATM
K00613 doeA
K00305 ectA
K00060 CMO
K00304 dsdA
K00303 kbl, GCAT
K00294 soxA
-3 -2 -1 0 1 2 3 4
log2 fold change
Kegg annotation category
KO ID number
Gene name


Fungus garden iron concentration (mg/kg)
7500
5000
2500
0
A. bisphaerica
A. capiguara
A. laevigata
A. sexdens
Grass-cutters
Dicot-cutters